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Inflammasome Priming in Sterile Inflammatory Disease

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The inflammasome is a cytoplasmic protein complex that processes interleukins (IL)-1β and IL-18, and drives a form of cell death known as proptosis. Oligomerization of this complex is actually the second step of activation, and a priming step must occur first. This involves transcriptional upregulation of pro-IL-1β, inflammasome sensor NLRP3, or the non-canonical inflammasome sensor caspase-11. An additional aspect of priming is the post-translational modification of particular inflammasome constituents. Priming is typically accomplished *in vitro* using a microbial Toll-like receptor (TLR) ligand. However, it is now clear that inflammasomes are activated during the progression of sterile inflammatory diseases such as atherosclerosis, metabolic disease, and neuroinflammatory disorders. Therefore, it is time to consider the endogenous factors and mechanisms that may prime the inflammasome in these conditions.

Innate Immunity and the Inflammasome

The innate immune system is specialized to perform receptor-mediated surveillance for pathogen and/or tissue damage. Pattern recognition receptors are the first line of defense against invading microbes and are expressed in a variety of immune cells including macrophages, epithelial cells, dendritic cells, neutrophils, and adaptive immune cells. Some of these PRRs, such as the TLRs and C-type lectins (CTLs), are expressed on the cell surface and can be activated directly via external pathological signals known as pathogen-associated molecular patterns (PAMPs). PRRs are also triggered during sterile inflammatory diseases, suggesting a crucial role for danger signals that are not foreign but are instead host-derived. These are known as danger-associated molecular patterns (DAMPs). Both PAMPs and

DAMPs can also make their way into the cell where they can trigger intracellular innate immune receptors directly, for example via recognition of DNA or RNA in the cytoplasm. Some cytosolic innate immune sensors are also activated indirectly as a result of a loss of homeostasis. These include NOD-like receptors (or NLRs) which assemble in the cytosol as inflammasomes, where they form a complex with the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) [1]. Inflammasomes regulate the processing of the protease caspase-1, which in turn regulates the cleavage of cytokines IL-1β and IL-18 from their respective precursors [2].

Of all the NLR inflammasomes, NLRP3 is the most widely studied [3, 4, 5]. The NLRP3 inflammasome is formed after indirect sensing of both non-sterile insults derived from pathogens (PAMPs) and sterile stressors (DAMPs). These range from bacterial products, mitochondrial DNA, viruses, and ATP to particulate matter such as crystals and amyloids [6, 7, 8]. Before a functional NLRP3 inflammasome can be formed, however, transcriptional and post-translational mechanisms must achieve a reasonable level of NLRP3 expression within the cell, and a suitable level of pro-IL-1β must also accumulate – this is a process known **as** inflammasome priming, or 'signal 1' (Figure 1, Key Figure). This is distinct from cellular priming of B or T cells when they encounter antigen for the first time, which involves a process of differentiation and maturation.

NLRP3 Inflammasome Priming: First Hit

The functional regulation of an active inflammasome in a cell is a 'two-hit' process. The 'first hit' requires a non-activating stimulus to promote the transcriptional expression of key components of the inflammasome. Across species, the expression of both NLRP3 and IL-1β is known to be transcriptionally regulated [9]. This is thought to prime the inflammasome before its activation by second stimuli (second hit). The 'second hit', either by the same and/or by additional stimuli, promotes the functional activity of the NLRP3 inflammasome [10]. Although most mammalian cells do not have a ready pool of IL-1β, IL-18 protein is expressed more constitutively [11], and thus may not have the same requirements for priming. Induction of active NLRP3 protein is also required to activate this inflammasome in most cell types; however, because as pathogen-associated factors that can accomplish this are well described, our interest is now diverted towards those host derived factors that may prime the inflammasome during sterile inflammatory diseases such as atherosclerosis, metabolic disease, and neuroinflammatory disorders. Indeed, many chronic inflammatory diseases have a clear role for NLRP3 activation, but the pathways that prime this functionality are not often discussed. Therefore, we now review endogenous factors that prime the NLRP3 inflammasome such as reactive oxygen species (ROS), oxidized low-density

lipoprotein (oxLDL), hypoxia, complement, amyloids and misfolded proteins with a view to chronic and also monogenic inflammatory diseases that are sterile in nature. We also extend our discussion to the non-canonical inflammasome which also has a similar requirement for priming as the NLRP3 inflammasome, albeit by different mechanisms.

Mechanisms of NLRP3 Priming

ROS Priming of the NLRP3 Inflammasome

The majority of the literature to date has focused on the role of the mitochondria and mitochondrial reactive oxygen species (mROS) as signal 2 required for the generation of mature IL-1β [12, 13, 14, 15]. An important distinction however is that activation of NLRP3 may actually be related to mitochondrial membrane potential [16, 17], while mROS production itself may in fact influence priming of NLRP3 [18]. For example, treatment of mouse macrophages with diphenyliodonium (DPI), a global mROS scavenger, was shown to impair lipopolysaccharide (LPS)-induced NLRP3 expression [5]. Furthermore, treatment with DPI decreased IL-1β and IL-18 production following inflammasome activation, but only when DPI was added before the priming step (signal 1), suggesting that ROS – possibly of mitochondrial origin – could potentially block inflammasome activation by impairing the priming step required for NLRP3 expression, and without affecting direct NLRP3 activation [5].

More recently, exposure of cells to hyperosmolarity has been demonstrated to induce NLRP3, ASC, pro-caspase-1, and pro-IL-1β expression and to promote the generation of ROS in human corneal epithelial cells [19]. These effects were abrogated when cells were treated with the antioxidant *N*-acetyl cysteine (NAC). The authors demonstrated that the induction of ROS following exposure to hyperosmotic stress preceded the effects on NLRP3, ASC, pro-caspase-1, and pro-IL-1β expression, further supporting ROS as a mediator of NLRP3 priming in these cells. Moreover, a study performed on patients suffering from dry eye, a disease associated with tear film hyperosmolarity and inflammation, demonstrated that ocular surface samples from these patients displayed elevated NLRP3, ASC, pro-caspase-1, and pro-IL-1β expression levels, as well as increased ROS levels compared to healthy controls [19]. This provides some evidence of a disease-relevant pathology where ROS may prime the NLRP3 inflammasome.

Of note, as described in the sections below, ROS has emerged as a common effector mechanism shared by NLRP3 priming in response to hypoxia, metabolites, and several other conditions, suggesting that ROS may be a principal component required to prime the NLRP3 inflammasome in general.

Hypoxia Priming of the NLRP3 Inflammasome

Hypoxia is known to promote pro-IL-1β production and has been implicated in the pathogenesis of several diseases including atherogenesis, where patient samples display increased NLRP3-derived IL-1β production [20]. This study demonstrated that hypoxia could augment NLRP3 gene expression and protein levels in both LPS-treated and untreated human macrophages *in vitro*, and promoted pro-IL-1β production by limiting its autophagic degradation[20].

In another study, animals subjected to renal ischemia/reperfusion (I/R) injury, which is known to induce hypoxia, displayed a significant upregulation of *Nlrp3* and *Pycard* (ASC) gene expression compared to sham-operated control mice [21]. *Nlrp3* RNA expression and protein levels were also increased in the brain following hypoxia–ischemia (HI) in a mouse model [22]. At earlier time points following HI, NLRP3 expression was detected in astrocytes in the hippocampus and the thalamus, and at later time points both astrocytes and microglia showed increased NLRP3 expression [22]. Furthermore, hypoxic exposure was found to increase the levels of NLRP3, AIM2, and pro-IL-1β in human prostate epithelial cells, in prostate cancer cell lines, and in THP-1 cells, and was targeted using an NF-κB inhibitor [23]. The authors suggested that such inflammasome priming might potentially contribute to prostatic chronic inflammation in human prostate tumors, but further validation is required.

Of note, the hypoxia-inducible factor 1α (HIF-1α), a global transcription factor governing responses to hypoxia, is an NF-κB-dependent gene, and the gene encoding NF-κB itself contains a hypoxia-response element (HRE) to which HIF-1α can bind to promote its expression [24]. Therefore, agents that activate NF-κB are likely to increase HIF-1α levels, and vice versa. Indeed, treatment of PC-3 human prostate cancer cell lines with two hypoxic mimetics known to stabilize HIF-1α, dimethyloxalylglycine (DMOG) and cobalt chloride ($CoCl_2$), boosted NLRP3 and pro-caspase-1 levels [25]. The authors propose, but do not confirm, a role for ROS in such hypoxia-regulated priming. By contrast, $CoCl_2$ was observed to have the opposite effect in mouse mixed glial cells [26]. Interestingly, both the human *NLRP3* and *PYCARD* (ASC) genes are predicted to contain a HIF-1α binding site. This implies that hypoxia could be a key pathway priming the inflammasome, and that there might be significant synergy with NF-κB-activating factors in this regard.

NLRP3 Priming by Metabolites

The concept that metabolites can regulate NLRP3 is intriguing given their imbalance in several diseases related to inflammasome activation. While this idea is only in its infancy, several lines of evidence suggest that several metabolites can influence inflammatory processes. For instance, dimethyl fumarate (DMF), a cell-permeable ester of the Krebs cycle metabolite fumarate, has recently been approved as the front-line drug for the treatment of relapsing-remitting multiple sclerosis [27]. Dimethyl fumarate has been suggested to exert its beneficial effects largely by decreasing neuroinflammation [28]. It has also been shown to decrease ROS production in a mouse macrophage cell line [29], most likely as a consequence of its upregulation of antioxidants such as heme oxygenase-1, superoxide dismutase-2, and nuclear factor erythroid 2-related factor 2 (Nrf2), as well as decreasing IL-1β (*Il1b*) mRNA expression, as shown in primary rat microglial cells [28]. Interestingly, DMF has been reported to be beneficial in the treatment of a mouse model of dextran sulfate sodium (DSS)-induced colitis by decreasing NLRP3 expression and activation in an Nrf2-dependent manner [30]. In agreement with the *in vivo* data, pretreatment of inflammasome-activated human monocytic THP-1 cells with DMF decreased mRNA levels of NLRP3 and pro-IL-1β [30]. This effect was also evident following inflammasome activation with alum and monosodium urate (MSU) crystals. The authors further demonstrated that DMF limited mitochondrial perturbations, including mROS production, mitochondrial membrane potential collapse, and mitochondrial DNA release, which occurred as a consequence of inflammasome activation. Nevertheless, whether DMF mediates its effects through this pathway to trigger NLRP3 priming specifically remains unclear.

More generally, alterations in the glycolytic pathway have also been implicated in NLRP3 priming, as studied in mouse and human myeloid cells. For example, 2-deoxyglucose (2DG) – a potent inhibitor of glycolysis – has been shown to suppress LPS-induced pro-IL-1β expression [31, 32, 33] and more recently has been found to limit ROS production in response to LPS and ATP, suggesting that its inhibitory effects may be at least partially ROS-dependent [32]. 2DG also inhibits NF-κB, and is thereby likely to impair NLRP3 expression and priming [34]. One study demonstrated that 2DG only inhibited IL-1β production when added before the LPS priming step, suggesting that it might limit pro-IL-1β and NLRP3 priming [35]. By comparison, other work showed that addition of 2DG after LPS, and before ATP, could affect NLRP3 inflammasome activation (signal 2) to generate mature IL-1β [32]. Unexpectedly, in the latter study 2DG treatment had no effect on NLRP3 levels. This could suggest a role for post-translational regulation of NLRP3 priming, as discussed below. Consequently, it is clear from these studies that glucose metabolism is required

for inflammasome function, but the data discriminating between NLRP3 priming and activation are controversial.

The most definitive example of metabolite-mediated priming of NLRP3 stems from a recent publication. It was observed that the metabolite itaconate, the levels of which are highly upregulated in mouse M1 macrophages, limited the induction of NLRP3, ASC, and pro-IL-1β in response to LPS, and subsequently impaired mature NLRP3-dependent IL-1β production *in vitro*[36]. In addition, RNA sequencing analysis revealed that itaconate pretreatment decreased the expression of several LPS-induced genes linked to inflammasome priming, including *Il1b*, *Il18*, *P2rx7*, *Casp1*, and *Pycard* (which codes for ASC). Furthermore, in the same system, itaconate was shown to limit LPS-induced ROS production, providing mechanistic evidence for its inhibition of IL-1β. This could help to explain why the same study also observed inhibition of a separate inflammasome nucleated by AIM2. Previously, overexpression of Irg1, the enzyme responsible for the generation of itaconate, was shown to increase the expression of *Tnfaip3* (coding for A20), a negative regulator of NF-κB, in LPS-tolerized mouse macrophages [37]. This may represent another means by which itaconate could limit NF-κB activity to impair NLPR3 expression and inflammasome priming.

Inflammasome Priming via Lipid Metabolism

Products of lipid metabolism have previously been identified as activators of the inflammasome, and different laboratories have demonstrated that both palmitate and cholesterol crystals can activate the NLRP3 inflammasome [38, 39]. As described below, it has become clear that there is also a role for lipid metabolism in inflammasome priming, and that the lipidome of a cell can either facilitate or diminish priming of the inflammasome.

Fatty acid synthesis is a major pathway in lipid metabolism, and the enzyme fatty acid synthase (FASN) produces palmitate from malonyl-CoA, acetyl-CoA, and NADPH. Recent work has demonstrated that functional FASN is essential for inflammasome priming in mouse peritoneal macrophages in response to LPS [40]. The authors demonstrated, through pharmacological and genetic approaches, that loss of function of FASN reduced NLRP3 induction in response to inflammatory stimuli, and that inhibition of FASN decreased IL-1β and IL-18 levels in serum of mice during LPS challenge. Upstream of this, the mitochondrial uncoupling protein-2 (UCP2) was found to regulate FASN, and thus NLRP3 priming in mouse macrophages. Deletion of UCP2

provided therapeutic benefit in a mouse model of sepsis involving cecal ligation and puncture (CLP). Downstream, FASN facilitated the inflammatory signal by promoting the phosphorylation of AKT; however, the kinase through which FASN might coordinate phosphorylation was not identified [40]. Activation of this kinase might occur through a regulatory lipid, downstream of FASN, or perhaps FASN might harbor an alternative novel enzymatic function. However, these questions remain unanswered.

Cholesterol metabolism is another major pathway in lipid metabolism that is linked to inflammasome priming. Liver X receptor (LXR) transcription factors are activated in response to high levels of cholesterol to reduce cholesterol levels. However, in response to low cholesterol levels, sterol regulatory element binding proteins (SREBPs) are activated to increase transcription of the genes involved in fatty acid and cholesterol synthesis [41, 42]. Similarly, while the activation of LXRs reduces cholesterol and negatively regulates *IL1B*transcription [43], activation of SREBPs not only increases cholesterol synthesis but also increases inflammasome priming, as shown in human umbilical vein endothelial cells (HUVEC) *in vitro*, and in mouse aortas *in vivo*[44]. In this study, oscillations in aortic blood flow over HUVECs not only promoted SREBP2 cleavage and activation but also promoted inflammasome priming by directly binding to the *NLPR3* promoter. This demonstrated that proteins required for cholesterol synthesis might also have the capacity to directly increase the expression of inflammasome constituents. In a transgenic mouse model overexpressing SREBP2 on an apolipoprotein E knockout (*Apoe*$^{-/-}$) background, the mice displayed increased atherosclerotic lesion size accompanied by increased expression of inflammasome components in the aortic arch [44]. This was associated with a disturbed flow pattern through the aortic arch, termed atheroprone flow, promoting activation of the overexpressed SREBP2 [44]. These data suggested that the initial trigger for atherosclerosis might be the shear force on the endothelium of the aorta activating SREBP2, and subsequently increasing priming of the inflammasome. Although these findings have not yet been replicated in macrophages or other immune cells, inflammasome priming might occur not only in response to pathogenic activators but, remarkably, also in response to physical stress.

Inflammasome Priming via Complement

The complement system is an integral part of the innate immune response (Box 1). Recent experimental evidence implicates the sublytic membrane attack complex (MAC) of complement in inflammation, modulating caspase-1 activation and IL-1β secretion [45]. In particular, it has been

demonstrated that sublytic MAC can trigger increased Ca^{2+} and, in turn, NLRP3 activation in primary human lung epithelial cells [45]. Moreover, MAC activation seems to be indispensable for NLRP3-mediated IL-1β production because mice lacking complement protein C6, which have an impaired terminal MAC pathway, are not able to activate the inflammasome [46]. In addition, targeted deletion of complement component 9 (C9) leads to decreased IL-1β production in a mouse model of LPS-induced inflammation, suggesting that C5b-9 might play a role in inflammasome priming or activation [47].

Recently, MAC has been suggested to provide both the priming and the activation signals for inflammasome activation because experiments indicated that MAC-mediated 'bystander' damage could lead to NLRP3 activation in mouse macrophages [48]. Specifically, this study showed that phagocytosis of complement-opsonized particles could promote inflammasome activation by a novel 'bystander' mechanism involving the transfer of MAC from the activating particle to a nearby cell. Of note, complement component C3a might function in this capacity because recent studies have implicated C3a in modulating IL-1β production in human monocytes [49, 50]. Specifically, these studies found that C3a binding to the C3aR could drive IL-1β production by triggering ERK1/2 activation, leading to increased ATP efflux, and activating in turn the purinoceptor **7** (P2X7) as well as the NLRP3 inflammasome. The activation of this pathway synergized with TLR4 stimulation, leading to enhanced type 17 T helper (Th17) cell responses; indeed, Th17 cell responses are relevant in pathogen clearance, and IL-17-mediated pathologies are associated with complement, as in the case of asthma [51].

Cholesterol crystals (CC) [52], MSU, and calcium phosphate crystals have all been found to trigger NLRP3 activation through lysosomal damage and ROS production [53]. The complement system has also been shown to recognize crystalline material, resulting in the production of C5a [54]. Recent studies have documented that C5a can trigger inflammasome activation in the presence of crystalline material [55, 56]. The first of these studies demonstrated that CC induced complement-dependent inflammasome activation via C5a in human peripheral blood mononuclear cells [55]. The second study clearly showed that C5a could act as an endogenous priming signal for inflammasome activation because C5a alone was able to trigger inflammasome signal 1 upon induction of pro-IL-1β in primary human monocytes [56]. Binding of C5a to its receptor, C5aR, can also lead to calcium mobilization and triggering of intracellular signaling cascades such as phospholipase C [57], phospholipase D [58], PI3K [59, 60], Raf-1-mediated activation of MEK-1 [61], PI3K, MAP/ERK kinase, and Akt [62], potentially representing another priming mechanism of NLRP3. C5a is also

able to prime human retinal pigment epithelial (RPE) cells for inflammasome activation in combination with lipofuscin[63], whose byproduct aggregates have been implicated in the pathogenesis of age-related macular degeneration (AMD). In a separate study, C5a was also shown to increase IL-1β production via the mitogen activated protein (MAP) kinase p38 through C5aR1, but only in monocytes [64]. This study found opposite effects of C5a on mouse macrophages, with inhibition of IL-1β production occurring independently of C5aR1. However, during LPS challenge of mice *in vivo*, C5aR1 deletion resulted in reduced IL-1β levels in the circulation, confirming the importance of C5aR1 in IL-1β induction, as shown in monocytes [64].

Post-Translational Modifications as Master Regulators of Transcription-Independent NLRP3 Priming

The idea of a rapid, transcriptional-independent, priming mechanism stems from the recent observation that inflammasome components undergo various different post-translational modifications. It was initially reported that inflammasome priming could occur as rapidly as 10 minutes following LPS treatment in murine macrophages, an event that did not require protein synthesis [65]. This study found NLRP3 to be in an ubiquitinated, inactive state in resting macrophages, and demonstrated that mROS was responsible for triggering its deubiquitination following priming with LPS [65]. The role of deubiquitination in inflammasome activation has been later confirmed by other studies [66, 67]. NLRP3 was also found to be in an inactive polyubiquitinated state, and the deubiquitinase BRCC3 (BRCA1/BRCA2-containing complex subunit 3) was identified as a responsible mediator for activation of the inflammasome following signal 2 [66]. Interestingly, not all deubiquitinase inhibitors block priming-mediated deubiquitination, and BRCC3 has specifically been shown to have no effect on priming in these studies [66, 67]. The deubiquitinase responsible for NLRP3 inflammasome priming thus remains to be identified. *In vivo*, ubiquitination may be regulated in a tissue-specific manner, with mouse macrophages experiencing high levels of IL-10 that promote ubiquitinated-NLRP3 turnover, and, because the macrophages were obtained from colonic tissue, this pathway may have relevant implications for the pathogenesis of inflammatory bowel disease [68, 69].

Another post-translational modification associated with NLRP3 inflammasome transcription-independent priming is phosphorylation. The extracellular signal-regulated kinase 1 (ERK-1) has been shown to be required in human monocytes for NLRP3 priming, while IL-1R-associated kinase (IRAK1) has been implicated in transcriptional-independent priming in murine macrophages [70, 71].

However, no phosphorylation site on NLRP3 has been associated with priming to date, and whether ERK and IRAK1 play a role in phosphorylating NLRP3 remains to be determined. Both these kinases − similarly to the NLRP3 deubiquitinase − depend on ROS production to exert their activities. A ROS scavenger has been found to block both IRAK1 and ERK effects on priming[70,71]. This emphasizes the importance of ROS in priming and the key role played by metabolism in immune cells driving inflammation. Lastly, it is interesting to speculate whether there might be a role for metabolism-derived post-translational modifications such as acetylation or malonylation in priming the NLRP3 inflammasome [72].

Priming the Inflammasome in Chronic Inflammatory Diseases

Metabolic Disorders and Atherosclerosis

The NLRP3 inflammasome is expressed in many metabolically relevant cell types such as macrophages [73], hepatocytes [74], and adipocytes [75]. The first study to demonstrate the relevance of the NLRP3 inflammasome in atherosclerosis showed that targeted disruption of individual components of the NLRP3 inflammasome (*Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ or *Casp1*$^{-/-}$) significantly protected proatherogenic mice from disease [52]. Furthermore, transplantation of *Nlrp3*$^{-/-}$ bone marrow into low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) recipient mice was sufficient to reduce lesion sizes and decrease circulating levels of IL-1β and IL-18 compared to mice receiving *Nlrp3*$^{+/+}$ bone marrow [52]. Collectively, these studies indicate that the NLRP3 inflammasome, present in hematopoietic cells, is required for atherosclerotic plaque progression in hypercholesterolemic murine models.

Increased circulating levels of low-density lipoprotein (LDL) are associated with atherosclerosis [76,77,78], obesity [79, 80], and insulin resistance [81]. Oxidized LDL is shown to provide both signal 1 and signal 2 to prime and activate NLRP3 inflammasome in mouse models of atherosclerosis [52] and islet amyloid polypeptide (IAPP)-induced inflammasome activation in type 2 diabetes (T2D) [31]. Furthermore, scavenger receptor CD36 has been implicated in the regulation of oxLDL-mediated NLRP3 inflammasome priming via TLR4/TLR6 in mouse macrophages and in *Apoe*$^{-/-}$ mouse models of atherosclerosis [82]. This provides evidence for a single DAMP being able to prime and activate the inflammasome to produce modest levels of IL-1β, consistent with the low-grade but chronic inflammatory condition occurring during obesity and atherosclerosis. Ablation of NF-κB family kinase IKKi in the hyperlipidemic, proatherogenic *Apoe*$^{-/-}$ mouse model has reportedly led to increased NLRP3 inflammasome priming [83]. This work shows that induction

of IKKi parallels priming of the NLRP3 inflammasome in PRR-activated mouse macrophages *in vitro*[83]. Once induced, it limits the chronicity of NLRP3 inflammasome priming and the subsequent inflammatory response in this proatherogenic and hyperlipidemic mouse model [83].

Given the heterogeneous cellular composition of atherosclerotic plaques, inflammasome regulation may not be limited to macrophages but could also occur in vascular smooth muscle cells (VSMCs). For example, expression of cytokines such as TNF-α in human atheroma plaques was shown not only to localize to macrophages but also to endothelial cells and VSMCs [84]. Indeed, cytokines such as TNFα can mediate inflammasome priming in human VSMCs by upregulating *IL1B* and *NLRP3* gene expression [85]. TNF production from mouse macrophages, or by foam cells in the context of atherosclerosis, could therefore act in a paracrine or autocrine fashion to prime the NLRP3 inflammasome [86]. NLRP3 silencing in VSMCs can therefore decrease IL-1β activation and secretion [85]. These studies suggest a role for the NLRP3 inflammasome in non-immune cells, and these might have a contributory effect in the progression of various chronic inflammatory pathologies.

Obesity-Linked Disease

Low-grade, chronic inflammation of adipose tissue is characterized by infiltration of proinflammatory macrophages, and both these features have been correlated with obesity-associated insulin resistance and NLRP3 activation [39, 75]. Furthermore, macrophage switching from an anti-inflammatory (M2 macrophage) to proinflammatory (M1) phenotype occurs in white adipose tissue (WAT) as the adipose tissue becomes more metabolically dysregulated [87]. Obesity-associated insulin resistance is characterized by increased expression of inflammasome-derived proinflammatory cytokines such as *Il1b* and *Nlrp3* in mice [75, 88]. Activation of NLRP3 activity in adipose tissue macrophages (ATMs) during diet-induced obesity may mediate insulin resistance in adipocytes via IL-1β secretion [39]. Secreted IL-1β is reported to disrupt the insulin signaling pathway, reduce the expression of insulin receptor substrate 1 (IRS1), and lead to decreased insulin-mediated glucose uptake resulting in insulin resistance [89, 90]. Secreted IL-1β can also lead to its transcriptional autoregulation by a positive feedback loop via NF-κB pathways [91], leading to amplification of the priming signal.

As mentioned above, TNF can also prime the NLRP3 inflammasome, and it has been shown that this is relevant for aged mice, which can develop increased NLRP3 expression in liver and adipose

tissue, associated with peripheral insulin resistance and impaired glucose tolerance [92]. Overall, the priming and subsequent activation of the NLRP3 inflammasome in macrophages infiltrating different tissues during metabolic stress can result in increased inflammation and metabolic dysregulation in mouse models of metabolic dysregulation as well as in human disease [93, 94].

During progressive obesity, adipose tissue expansion can become limited, and the surplus spill-over of fat into the circulation and non-adipose organs during obesity has been suggested as a pathogenic mechanism [95]. This fat leakage may be scavenged by ATMs, resulting in ceramide synthesis. Ceramide has been reported to activate the inflammasome in mouse macrophages, resulting in increased inflammation in WAT during obesity [75]. Based on the observation that ceramides drive other proinflammatory pathways, such as NF-κB, this could be an endogenous priming agent for the inflammasome as well [96]. More evidence surrounds activation of the inflammasome by saturated fatty acids (SFAs) that are also liberated into the circulation during obesity [97]. These are thought to trigger TLR pathways in mice, and thus may also prime the NLRP3 inflammasome in the context of T2D [98], and could also play a role in non-alcoholic fatty liver disease (NAFLD) [99] as well as in fibrotic kidney disease. Although some reports did not find an effect of SFAs on NLRP3 priming [97], an activating *Nlrp3* genetic mutation in mice that does not affect priming has been shown to lead to an additive effect with diets contributing to NAFLD [100]. This suggests that dietary intake with effects on metabolism could have a significant role in NLRP3 inflammasome priming.

Neuroinflammation

Amyloids and misfolded proteins are frequently associated with activation of the NLRP3 inflammasome *in vivo*[101]. In some cases the misfolded protein itself may not only act as signal 2, to trigger NLRP3 oligomerization, but may also be able to act as signal 1, to prime NLRP3 and IL-1β expression. This is not necessarily a requirement, however, because complex biological processes are usually required to establish large quantities of amyloids or misfolded proteins *in vivo*, and these same processes can liberate other inflammatory molecules to prime the inflammasome. We highlight some examples of both settings, where inflammasome priming is either directly or indirectly associated with amyloids and misfolded proteins.

One of the most profound *in vivo* effects of *Nlrp3* deletion comes from a mouse model of Alzheimer's disease. In this model familial forms of mutant amyloid precursor protein (APP) and

presenilin 1 (PS1) are overexpressed in mice, and deletion of *Nlrp3* has been shown to significantly improve object and spatial memory [102]. *In vitro* data using mouse macrophages suggest that amyloid can not only act as signal 2 to activate the inflammasome but may also engage with TLR4 to trigger NF-κB and prime the inflammasome [103]. This is true not only for the Aβ amyloid associated with Alzheimer's disease but also for the IAPP amyloid that forms deposits in the pancreas during T2D [104], although this may proceed through TLR2/6 [105]. However, the effect of amyloids engaging TLRs has not been universally reported by all laboratories [31], hypothetically because of differences in the size of amyloid oligomers used in different experiments. Speculating further, *in vivo* it is possible that amyloidogenic species may bind to other DAMPs to facilitate activation of TLRs or of other NF-κB-dependent signaling pathways to prime NLRP3 inflammasome activity. Another amyloid species, drusen, forms deposits in the eyes of individuals suffering from acute macular degeneration (AMD). These deposits are commonly associated with complement, which could act as a priming factor as discussed above. All amyloid-associated diseases also feature accumulation of advanced glycation end-products (AGEs) and, through their receptor RAGE, these could also prime the NLRP3 inflammasome during chronic amyloid-related pathology [106].

Although not an amyloid, mutant superoxide dismutase 1 (SOD1) is another misfolded protein that has been reported to trigger the NLRP3 inflammasome in familial amyotrophic lateral sclerosis [107]. In this case, as a cytoplasmic protein, there is less capacity for misfolded SOD1 to escape extracellularly and trigger TLR priming of the inflammasome in bystander cells. However, misfolded proteins can also lead to endoplasmic reticulum (ER) stress intracellularly [108], which could trigger signal 1 for NLRP3 activation [109], perhaps via damaged mitochondria and ROS [110]. S100A8/A9 proteins and HMGB1 are also molecules released by dead or dying cells downstream of an accumulation of misfolded proteins, and these also have the capacity to prime the inflammasome, as shown using peripheral blood mononuclear cells *in vitro*[111] and in mouse models [112]. This is also likely to be true for mutant α**-synuclein** protein, that is associated with Parkinson's disease, which has been reported to engage TLR2 on human monocytes to prime the NLRP3 inflammasome [113].

Human pathology associated with amyloids and misfolded proteins frequently features elevated levels of serum AGE and S100A8/A9 proteins, rendering them attractive candidates for inflammasome priming during these, and perhaps other, chronic neurodegenerative conditions.

Nevertheless, the field still needs to validate many of these findings, seeking to explain the discrepancies mentioned.

Monogenic Autoinflammatory Disease

Mutations that constitutively activate NLRP3 are one type of monogenic autoinflammatory disease**.** These periodic fever syndromes result in spontaneous 'signal 2' driving inflammasome activation, but still require priming to trigger the inflammatory pathology [114]. Nevertheless, this disease is dominantly inherited, and therefore priming must somehow be triggered during the lives of these patients, either from exposure to the environment, the microbiome, or endogenous DAMPs. Mice with similar mutations in NLRP3 display delayed disease progression when treated with antibiotics, suggesting not only that microbial PAMPs may contribute to priming *in vivo* but also that endogenous DAMPs might be potentially present because these mice do not resolve the disease [115].

Another autoinflammatory disorder where priming of the NLRP3 inflammasome may play a significant role is caused by loss-of-function mutations in the negative regulator of NF-κB A20 (HA20, haploinsufficiency of A20) [116]. Increased inflammation driven by constitutively active NF-κB in patients does not seem to result in a significant increase in IL-1β or IL-18 unless their cells are treated with LPS [116]. NLRP3 expression appears to be increased and therefore primed, as shown in the mouse model of A20 deficiency, where deletion of *Nlrp3* can completely protect mice from disease [117]. Perhaps differences in mice and humans in this instance may reveal different priming mechanisms that are difficult to compare, with humans being exposed to a vast array of microbes relative to laboratory mice.

These monogenic conditions highlight diseases that are not associated with infection, but nevertheless are NLRP3-dependent, and may require both microbial and endogenous priming.

Priming of the Non-Canonical Inflammasome

For decades, the protection of TLR4 mutant mice against LPS lethality was a cornerstone for innate immunity. Remarkably, the laboratories of Dixit and Miao independently rewrote this dogma and showed that, when mice were first primed to induce the non-canonical caspase-11 inflammasome, they were then susceptible to LPS lethality even in the absence of TLR4 [118, 119]. Caspase-11 can directly detect cytosolic LPS and, intriguingly, this also requires a priming step [120]. Whereas

NLRP3 inflammasome priming for canonical caspase-1 inflammasomes is mediated by the TLR4/MyD88 axis, priming of non-canonical caspase-11 upon *Escherichia coli* or *Citrobacter rodentium* challenge is mediated by TLR4/TRIF [121, 122, 123]. Principally, this seems to be due to a requirement for interferon (IFN) signaling to induce caspase-11 expression (Figure 2). Hence, type I IFNs seem to play a prominent role in the regulation of caspase-11 transcription. Caspase-11 expression is also upregulated by IFN-γ [124]. This requires NF-κB and Stat1 binding to the caspase-11 promoter [125]. There is also emerging literature suggesting that additional IFN-induced genes, guanylate-binding proteins (GBPs) and IRGB10, are required for LPS release from bacteria into the cytoplasm [126].

Surprisingly, despite the regulation of caspase-11 priming by IFN signaling, the potential of cellular metabolic processes and DAMPS being able to prime the non-canonical caspase-11 remain poorly studied. One study reported that ROS are able to induce caspase-11 during infection with *C. rodentium*[127]. A second study showed that, similarly to NLRP3, complement might also prime caspase-11 activity, although via a separate mechanism in which complement-related peptidase Cbp1 (carboxypeptidase B1) cleaves C3 to trigger C3aR [128]. Fas-associated protein with death domain (FADD) and caspase-8 in TLR4–NF-κB have also been proposed to prime both canonical and non-canonical inflammasomes [129]. This is intriguing, given the profound caspase-8-driven gut pathology that is associated with necroptosis of intestinal epithelial cells, which might connect the necroptotic and pyroptotic pathways implicated in IBD and sepsis in association with Gram-negative bacteria [130].

One might speculate on how all of this information might translate to human caspases 4 and 5 which are the homologs of murine caspase-11. In fact, most caspase-11 functionality appears to be encoded by caspase-4, which is constitutively expressed in myeloid cells [120]. Because caspase-5 is highly inducible, it may act more like caspase-11 with respect to priming; however, the specific mechanisms by which it is activated during a live infection remain to be elucidated [131]. The cellular distribution of caspase-4 and caspase-5 could also be a crucial determinant of their physiological functions. This information is important when considering how to intervene in the clinic for diseases associated with Gram-negative bacteria. Interestingly, small molecules with histone deacetylase activity appear to prevent caspase-11 expression and might be explored as putative therapeutic candidates [132]. One of these molecules, sodium butyrate, is known to bear

anti-inflammatory activity in the regulation of the gut microbiota in mouse models [133], consistent with the newfound role of caspase-11 in the detection of cytoplasmic LPS.

These studies highlight the importance of priming of the non-canonical inflammasome, bearing most relevance with regard to infections associated with Gram-negative bacteria. It is not yet clear which of these non-canonical inflammasomes are important in sterile inflammatory diseases; however, one interesting candidate may be metabolic disease. Indeed, oxPAPC (oxidized phospholipid) species may constitute a structural mimetic of LPS and directly bind to caspase-11 [134]. More data are required to validate this in relevant metabolic models where oxPAPC may be pathogenic, and these might then highlight sterile inflammatory conditions for which priming of non-canonical inflammasomes is mediated by endogenous factors through IFN.

Concluding Remarks

Many inflammasome pathways play an important function in the host response to microbial pathogens, but dysregulation of the NLRP3 inflammasome is associated with many important diseases, and its role in infectious disease is, perhaps, less important than for some other inflammasome pathways. Pharmacological inhibition of dysregulated inflammasome activation may provide much-needed therapeutic benefit for patients suffering from chronic inflammatory diseases (see Outstanding Questions and Box 2). With numerous promising inhibitors of signal 2 under development, the list of anti-inflammatory agents could be bolstered by ongoing work targeting pathways that trigger signal 1. Clear mechanistic insight on priming and the initial steps in inflammasome activation are likely to further our understanding of the pathogenic role of the inflammasome in various sterile inflammatory diseases. Furthermore, in this exciting field, such information may uncover clear opportunities for the development of novel therapeutic interventions to treat such conditions in the clinic.

Trends

NLRP3 inflammasome priming requires transcriptional upregulation of NLRP3 and pro-IL-1β expression, together with post-translational modification of NLRP3 itself.

Obesity and other chronic inflammatory states prime the NLRP3 inflammasome, and these constitute serious predisposing factors for inflammasome activation in sterile inflammatory disease.

Microbial priming of the inflammasome could potentially drive crosstalk between the environment and the development of autoinflammatory disease.

Inflammasome priming is not limited to immune cells, and can contribute significantly to overall pathology during chronic, low-grade inflammation.

Outstanding Questions

Is ROS the lynchpin of NLRP3 inflammasome priming? There are many paths towards priming the NLRP3 inflammasome, and many of them seem to converge on ROS. However, signal 2 of inflammasome activation is also implicated downstream of ROS. Therefore, delineating the separate pathways by which each of these processes occur will be challenging.

Which post-translational modifications of NLRP3 or other inflammasomes are required for activation? A two-step mechanism of inflammasome activation is a sensible safety measure; consequently, will post-translational modification(s) constitute a priming requirement for inflammasomes other than NLRP3 that do not require transcriptional upregulation? Other than ubiquitination and phosphorylation, many options exist.

Can NLRP3 inflammasome priming be targeted therapeutically? Targeting the priming step rather than the activation step may have broader effects on other inflammatory processes that could induce a favorable disease-modifying profile or unwanted susceptibilities to infection.

Is non-canonical inflammasome priming crucial for human caspase-4 or caspase-5 *in vivo*? This may not be important during Gram-negative bacterial infections, where priming will certainly occur, but if caspase-4 or caspase-5 can be implicated in sterile inflammatory disease in will be interesting to identify the factors that prime this process.

Box 1
The Complement System

The complement system comprises at least 30 soluble proteins, cell-surface receptors, and regulators which can be sequentially activated by three distinct pathways – the classical, the lectin, and the alternative cascades. Once activated, complement has the capacity to kill pathogenic microorganisms by inserting the terminal complement complex C5b-9 into the surface of the target cell, forming a lytic pore that destroy the membrane integrity of the microorganism [135]. The terminal complement membrane attack complex (MAC) is formed by the activation of C5, followed by sequential activation of C6, C7, C8, and the polymerization of C9 forming the lytic pore [136]. Although MAC can form a lytic pore on the cell surface of host cells, most nucleated cells have efficient repair mechanisms to prevent host cell lysis via the use of regulators and ion pumps, and trigger an inflammatory response [137].

Box 2
Clinician's Corner

Inhibition of Inflammasome Priming to Treat Inflammatory Diseases
The inflammasome pathway represents a promising target for a variety of autoimmune and auto-inflammatory diseases. There are many excellent recent reviews highlighting the discovery and novel mechanistic understanding of small-molecule inhibitors for NLRs and caspases, and the preclinical/ clinical impact of targeting the inflammasome products IL-1β and IL-18 in disease [138, 139, 140, 141, 142].

Interfering with inflammasome priming may represent a novel approach for treating various pathologies including inflammatory, metabolic, and infectious diseases as well as cancer. Targeting the expression levels of NLRP3 could potentially lead to more attractive long-term therapeutic modalities and approaches such as microRNA- and ubiquitination-based techniques could be envisaged.

Recent discoveries of several microRNAs (miRNAs) have shed light on the mechanistic regulation of NLRP3 expression levels through modulation of NLRP3 transcripts, and this might be therapeutically exploited to inhibit NLRP3 inflammasome activity. For example, miRNA-223 has been shown to suppress NLRP3 priming by binding to a conserved site in the 3′-untranslated region (UTR) of the NLRP3 transcript, thereby reducing downstream signaling [145, 146, 147]. In addition, inhibition of several other miRNAs involved in NLRP3 activation, miR115, miR133a-1, and miR-377, may be beneficial for suppression of NLRP3 function [148, 149, 150].

Modulating post-translational modifications of NLRP3 could also lead to advanced therapies. For example, non-transcriptional priming associated with NLRP3 activation involves rapid deubiquitination of the protein [151]. The use of G5, a deubiquitinating enzyme (DUB) inhibitor (without known selectivity), or knockdown of BRCCA, a DUB regulating NLRP3 activation, has been shown to inhibit NLRP3 function in mouse macrophages [66]. Moreover, treatment with the DUB inhibitor PR619 has been shown to attenuate IL-1β production by BMDMs, which may involve enhanced association to HDAC6, another negative regulator of NLRP3 function [152]. In addition, heterobifunctional small molecules that simultaneously bind to a target protein and a ubiquitin ligase, PROTACs, can be used to enable ubiquitination and subsequent degradation of a target (reviewed in [153]). Consequently, it may be possible to enhance NLRP3 ubiquitination and promote its protein degradation via PROTACs, a putative strategy for targeted deletion of NLRP3.

Various microbial ligands and inflammatory cytokines such as TNF-α and IL-1β positively regulate inflammasome priming through the induction of NF-κB-mediated NLRP3 and pro-IL-1β expression [91, 154, 155]. Inhibition of NF-κB signaling could thus represent one targeted approach to inhibit inflammasome activation. One molecule that may accomplish this, through effects on A20 and TRAF6, is γ-tocotrienol, as demonstrated in a mouse model of T2D [156].

Glossary

Apolipoprotein E knockout (*Apoe*$^{-/-}$) mice - Mice with a genetic deficiency for ApoE that is required for normal catabolism of triglyceride-rich lipoproteins.

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) - an adaptor protein used by many cytoplasmic PRRs to engage with caspase-1 in an inflammasome complex.

Atherogenesis - the formation of abnormal lipid masses in the arterial wall that are associated with immune cell infiltration.

Autophagic degradation - a cell-intrinsic mechanism to catabolize the endogenous components of a components.

Danger-associated molecular patterns (DAMPs) - direct ligands for PRRs that are generated by dead or dying cells.

Hypoxia - decreased oxygen availability.

Inflammasome priming - increasing NLRP3 and pro-IL-1β expression to a level that is sufficient for oligomerization and activation to proceed if triggered.

Itaconatea - metabolite with antibacterial effects; produced in macrophages.

Purinoceptor 7 (P2X7) - one of a family of cell-surface receptors triggered by ATP.

Lipofuscin - pigment granule composed of lipids that accumulate in lysosomes.

M1 macrophage - an ‘inflammatory’ macrophage subtype; it can be generated by stimulation with LPS and IFN-γ.

M2 macrophage - an ‘anti-inflammatory’ macrophage subtype (or ‘activated’); can be generated by stimulation with IL-4.

Pathogen-associated molecular patterns (PAMPs) - direct ligands for PRRs that are of microbial origin, otherwise known as MAMPs.

Pattern recognition receptors (PRRs) - receptors for ligands or signals that activate immune pathways.

Monogenic autoinflammatory disease - an inherited condition associated with inflammatory manifestations that can be periodic in nature.

Necroptotic and pyroptotic pathways - programmed cell death mediated by mixed-lineage kinase domain-like (MLKL; necroptosis) or caspase substrate gasdermin D (GSDMD; pyroptosis).

Non-canonical inflammasome - the executioner caspase of the non-canonical inflammasome is caspase-11 (mouse) or caspase-4/5 (human) rather than the canonical inflammasome (caspase-1).

Scavenger receptor CD36 - cell-surface import receptor for ligands such as low-density lipoprotein (LDL).

Sterile inflammatory diseases - inflammatory conditions that are not associated with a pathogenic infection

Sterol regulatory element binding proteins (SREBPs) - transcription factors that bind to DNA motifs commonly associated with enzymes regulating sterol biosynthesis.

α-Synuclein - a protein found as insoluble deposits in the brains of individuals with Parkinson’s disease.

Type 17 T helper (Th17) cell - inflammatory T helper cell defined by the production of IL-17.

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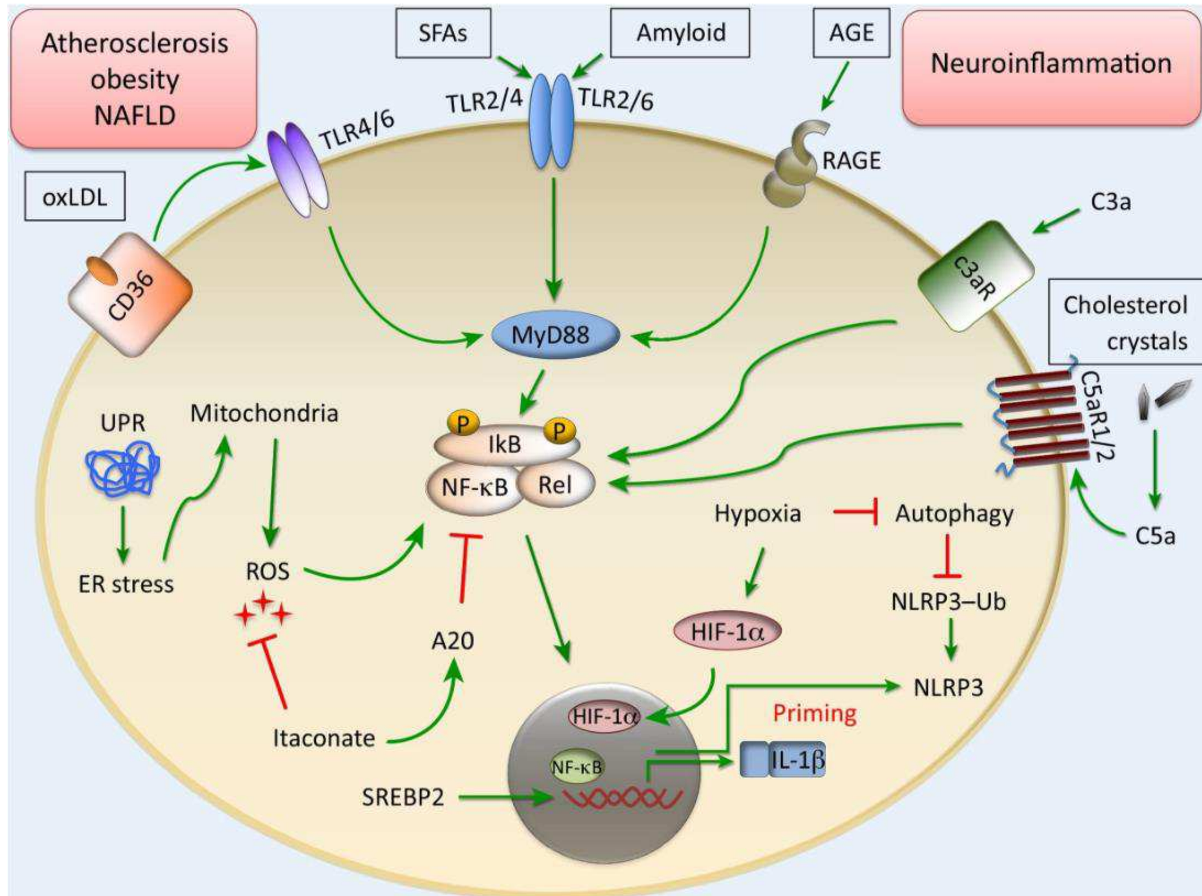
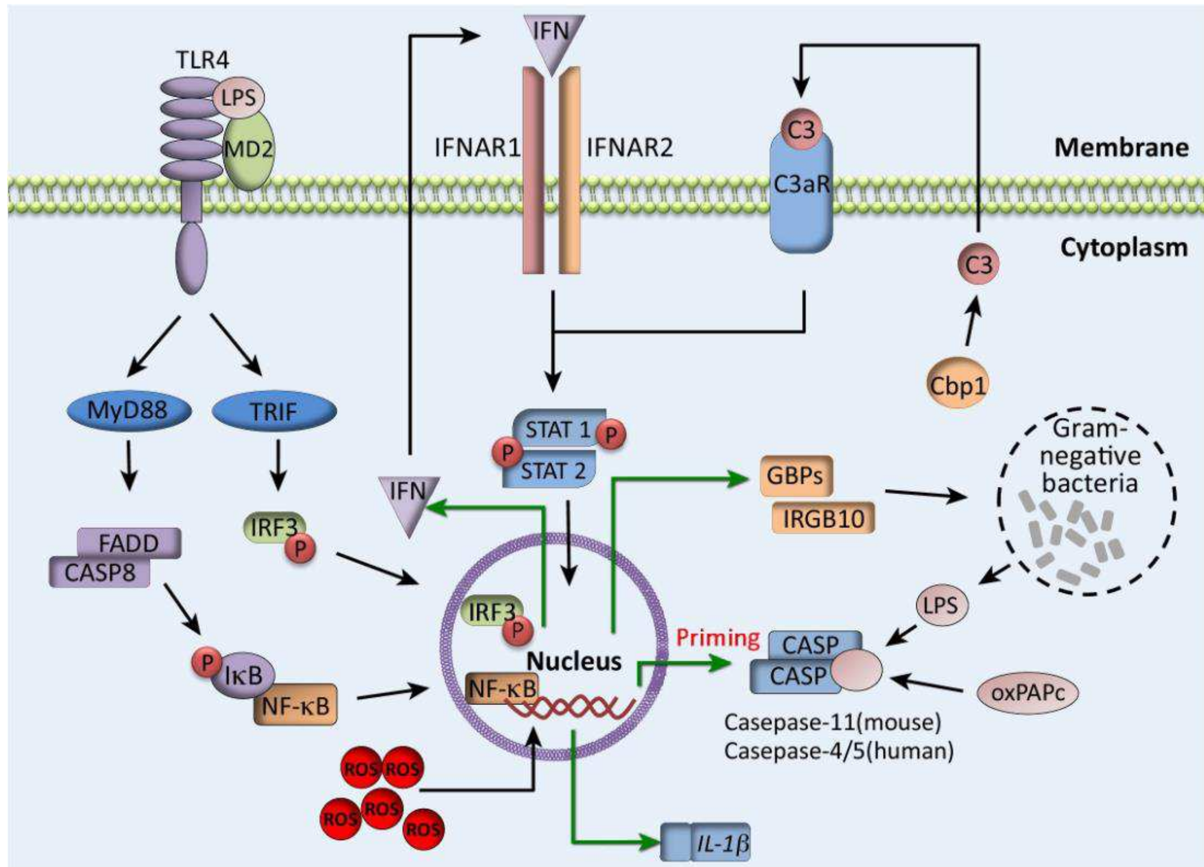
Figure 1.
Key Figure: Priming of the Canonical Inflammasome



Several different receptors can prime IL-1β and NLRP3 expression within the cell in response to endogenous DAMPs such as oxLDL, SFAs, amyloids, AGEs, and cholesterol crystals. Intracellular processes such as ER stress and metabolites such as itaconate also have pathways by which NLRP3 priming can proceed. Post-transcriptional and autophagic regulation of the expression of different inflammasome constituents may also be an important consideration in priming. Abbreviations: AGE, advanced glycation end-product; DAMPS, danger-associated molecular patterns; ER, endoplasmic reticulum; NAFLD, non-alcoholic fatty liver disease; oxLDL, oxidized low-density lipoprotein; P, phosphorylation; ROS, reactive oxygen species, SFAs, saturated fatty acids; Ub, ubiquitin; UPR, unfolded protein response.

Figure 2.



Priming of the Non-Canonical Inflammasome. Interferon production and complement activation induce the expression of caspases 11, 4, and 5 for non-canonical inflammasome activation. TRIF signaling also participates in priming and, together with Myd88 and reactive oxygen species (ROS), is important for the induction of pro-IL-1β which is necessary before it can be cleaved by caspases 11, 4, or 5. GBPs and IRGB10 are important for the liberation of lipopolysaccharide (LPS) from cytoplasmic vacuoles, while metabolic disorders may liberate oxidized phospholipid (oxPAPC) that can also directly bind to the non-canonical inflammasome sensors caspases 11, 4, and 5. Abbreviations: CASP, caspase; P, phosphorylation.